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(54) Title: NUCLEOTIDE DETECTION METHOD**(57) Abstract**

Provided is a method of identifying a selected nucleotide in a first nucleic acid utilizing a mobile solid support, as well as a novel read-out method for improving the use of mobile solid support-based read-out technologies for detection of nucleic acid polymorphisms in a target nucleic acid.

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Nucleotide Detection Method

Background

Field of the Invention

5 The present invention provides methods for rapid detection of single nucleotide polymorphisms (SNPs) in a nucleic acid sample. The present invention further provides a novel read-out method for improving the use of mobile solid support-based read-out technologies for detection of nucleic acid polymorphisms in a target nucleic acid. The methods can be utilized to detect SNPs in genomic DNA as
10 well as amplified DNA, RNA, etc., thus making them useful for a variety of purposes, including genotyping (such as for disease mutation detection and for parentage determinations in humans and other animals), pathogen detection and identification, and differential gene expression. The present invention further provides a method for identifying a nucleic acid utilizing a run-off sequencing reaction of a relatively short
15 portion of the nucleic acid. The method can be utilized, for example, to identify an EST from only a small portion of the EST and in an analysis of nucleotide polymorphisms. The reactions can be multiplexed to increase data readout capacity.

Background Art

20 Methods of detecting single base polymorphisms have typically involved hybridization reactions. For example, the method of performing a Luminex FlowMetrix-based SNP analysis involves differential hybridization of a PCR product to two differently-colored FACS-analyzable beads. The FlowMetrix system currently consists of uniformly-sized 5 micron polystyrene-divinylbenzene beads stained in
25 eight concentrations of two dyes (orange and red). The matrix of the two dyes in eight concentrations allows for 64 differently-colored beads (8^2) that can each be differentiated by a FACScalibur suitably modified with the Luminex PC computer board. In the Luminex SNP analysis, covalently-linked to a bead is a short

(approximately 18-20 bases) "target" oligodeoxynucleotide (oligo). The nucleotide positioned at the center of the target oligo encodes the polymorphic base. A pair of beads are synthesized; each bead of the pair has attached to it one of the polymorphic oligonucleotides. A PCR of the region of DNA surrounding the to-be analyzed SNP 5 is performed to generate a PCR product. Conditions are established that allow hybridization of the PCR product preferentially to the bead on which is encoded the precise complement. In one format ("without competitor"), the PCR product itself incorporates a fluorescein dye and it is the gain of the fluorescein stain on the bead, as measured during the FACScalibur run, that indicates hybridization. In a second 10 format ("with competitor,") the beads are hybridized with a competitor to the PCR product. The competitor itself in this case is labeled with fluorescein. And it is the loss of the fluorescein by displacement by unlabeled PCR product that indicates successful hybridization. It has been stated that "with competitor" is more discriminating in SNP analysis.

15 A method for typing single nucleotide polymorphisms in DNA, labeled Genetic Bit Analysis (GBA) has been described [Genetic Bit Analysis: a solid phase method for typing single nucleotide polymorphisms. Nikiforov T T; Rendle R B; Goelet P; Rogers Y H; Kotewicz M L; Anderson S; Trainor G L; Knapp M R. NUCLEIC ACIDS RESEARCH, (1994) 22 (20) 4167-75]. In this method, specific fragments of 20 genomic DNA containing the polymorphic site(s) are first amplified by the polymerase chain reaction (PCR) using one regular and one phosphorothioate-modified primer. The double-stranded PCR product is rendered single-stranded by treatment with the enzyme T7 gene 6 exonuclease, and captured onto individual wells of a 96 well polystyrene plate by hybridization to an immobilized oligonucleotide

primer. This primer is designed to hybridize to the single-stranded target DNA immediately adjacent from the polymorphic site of interest. Using the Klenow fragment of *E. coli* DNA polymerase I or the modified T7 DNA polymerase (Sequenase), the 3' end of the capture oligonucleotide is extended by one base using a mixture of one biotin-labeled, one fluorescein-labeled, and two unlabeled dideoxynucleoside triphosphates. Antibody conjugates of alkaline phosphatase and horseradish peroxidase are then used to determine the nature of the extended base in an ELISA format. This paper also describes biochemical features of this method in detail. A semi-automated version of the method, which is called Genetic Bit Analysis (GBA), is being used on a large scale for the parentage verification of thoroughbred horses using a predetermined set of 26 diallelic polymorphisms in the equine genome. Additionally, minisequencing with immobilized primers has been utilized for detection of mutations in PCR products [Minisequencing: A Specific Tool for DNA Analysis and Diagnostics on Oligonucleotide Arrays. Pastinen, T. et al. Genome Research 7:606-614 (1997)].

The effect of phosphorothioate bonds on the hydrolytic activity of the 5'-->3' double-strand-specific T7 gene 6 exonuclease in order to improve upon GBA was studied [The use of phosphorothioate primers and exonuclease hydrolysis for the preparation of single-stranded PCR products and their detection by solid-phase hybridization. Nikiforov T T; Rendle R B; Kotewicz M L; Rogers Y H. PCR METHODS AND APPLICATIONS, (1994) 3 (5) 285-91]. Double-stranded DNA substrates containing one phosphorothioate residue at the 5' end were found to be hydrolyzed by this enzyme as efficiently as unmodified ones. The enzyme activity was, however, completely inhibited by the presence of four phosphorothioates. On the

basis of these results, a method for the conversion of double-stranded PCR products into full-length, single-stranded DNA fragments was developed. In this method, one of the PCR primers contains four phosphorothioates at its 5' end, and the opposite strand primer is unmodified. Following the amplification, the double-stranded product 5 is treated with T7 gene 6 exonuclease. The phosphorothioated strand is protected from the action of this enzyme, whereas the opposite strand is hydrolyzed. When the phosphorothioated PCR primer is 5' biotinylated, the single-stranded PCR product can be easily detected colorimetrically after hybridization to an oligonucleotide probe immobilized on a microtiter plate. A simple and efficient method for the 10 immobilization of relatively short oligonucleotides to microtiter plates with a hydrophilic surface in the presence of salt was also described.

DNA analysis based on template hybridization (or hybridization plus enzymic processing) to an array of surface-bound oligonucleotides is well suited for high density, parallel, low cost and automatable processing [Fluorescence detection applied 15 to non-electrophoretic DNA diagnostics on oligonucleotide arrays. Ives, Jeffrey T.; Rogers, Yu Hui; Bogdanov, Valery L.; Huang, Eric Z.; Boyce-Jacino, Michael; Goelet, Philip L.L.C., Proc. SPIE-Int. Soc. Opt. Eng., 2680 (Ultrasensitive Biochemical Diagnostics), 258-269 (1996)]. Direct fluorescence detection of labeled DNA provides the benefits of linearity, large dynamic range, multianalyte detection, 20 processing simplicity and safe handling at reasonable cost. The Molecular Tool Corporation has applied a proprietary enzymatic method of solid phase genotyping to DNA processing in 96-well plates and glass microscope slides. Detecting the fluor-labeled GBA dideoxynucleotides requires a detection limit of approx. 100 mols./ μm^2 . Commercially available plate readers detect about 1000 mols./ μm^2 , and an

experimental setup with an argon laser and thermoelectrically-cooled CCD can detect approximately 1 order of magnitude less signal. The current limit is due to glass fluorescence. Dideoxynucleotides labeled with fluorescein, eosin, tetramethylrhodamine, Lissamine and Texas Red have been characterized, and 5 photobleaching, quenching and indirect detection with fluorogenic substrates have been investigated.

Although SNP analysis by hybridization is a powerful method, it has several disadvantages. These include; i) a need to synthesize two targets, and possibly two competitor oligonucleotides for each allelic pair, ii) the establishment of the 10 hybridization parameters (buffer content, temperature, time) that will efficiently discriminate between alleles, and iii) an avoidance of regions containing secondary structure that may effect the hybridization parameters.

Current limitations to the GBA methods as described include i) the limited density that can be achieved on a 2-dimensional solid surface, ii) photobleaching, iii) 15 autofluorescence of glass and plastic substrates, iv) difficulty in consistently coupling oligonucleotides to glass, and v) the expense, ease and flexibility of the system for creating new fixed arrays.

The present invention provides a novel system for using a GBA single base chain extension (SBCE) which takes advantage of the powerful matrixing capabilities 20 of a mobile solid support system having multiple dye color/concentration capabilities (e.g., the FlowMetrix system) to overcome the described disadvantages. The present invention further provides a method to improve the detection of reaction products from such polymorphism identification methods. Various detection methods, as described herein and as known in the art, can be enhanced by utilizing the present

detection method. Such methods can be combined with the present invention to provide a read out format that is time- and cost-efficient as it provides a means of using any given bead for use, individually, with many primers. This read-out method can be utilized also with many polymorphism detection methods, such as SBCE, OLA and cleavase reaction/ signal release (Invader methods, Third Wave Technologies).

Detailed Description

The present invention provides a method whereby a mobile solid support, such as a bead, which is detectably tagged, such as with a dye, a radiolabel, a magnetic tag, or a Quantum Dot® (Quantum Dot Corp.), is utilized in a nucleic acid read out procedure, either a direct readout onto a mobile solid support-linked nucleic acid such as SBCE, OLA or cleavase reaction/signal release (Invader methods, Third Wave Technologies, Madison, Wisconsin) or an indirect readout (in solution) which is then captured by an intermediate nucleic acid such as by a zipcode attached to a mobile solid support, and the readout product is then analyzed on a selected platform, such as by passing the mobile solid support over a detector (such as a laser detection device) or by passing a detector over the mobile solid support.

The present invention provides a novel system for SNP readout using an encoded mobile solid support which takes advantage of the powerful matrixing capabilities of a mobile solid support system. In one embodiment, the system uses a GBA single base chain extension (SBCE). In another embodiment, the system utilizes an oligonucleotide ligation assay. In yet another embodiment, the system uses an enzymatic or chemical read-out method whereby an enzyme or chemical is used to modify or endonucleolytically cleave a mismatched base at the polymorphic site, resulting in the loss of an attached reporter or said modification resulting in a labeling

means for the identification of the modification. Thus, in a further embodiment, the system utilizes an endonuclease cleavase/signal release method (Invader methods, Third Wave Technologies) (see, e.g., Marshall et al. *J. Clin. Microbiol.* 35(12):3156-3162 (1997); Brow, et al. *J. Clin. Microbiol.* 34(12):3129-3137 (1997)). In another 5 embodiment, fluorescence energy transfer (FET) is used with fluorescence quenching as a readout.

In the cleavase enzyme readout, target nucleic acid (e.g., PCR product or genomic DNA) hybridizes to both a complementary Invader probe and a Signal probe; a cleavase enzyme recognizes the specific structure formed between the target 10 nucleic acid, Invader probe, and Signal probe, and cleaves the Signal probe at the branch site and thereby releases the signal for detection. Another Signal probe can then bind to the nucleic acid and the cleavase reaction begins anew. This process is repeated many times and thereby increases the signal amplification. The essence for 15 cleavase to work is the presence of an overlapping base of the Invader probe with the signal base. In an improved version, named Invader Squared, two rounds of Invader are performed simultaneously. The primary invader reaction involves using SNP-specific target DNA, the resulting cleavase-product becomes functional in a secondary Invader reaction with a universal signal probe and universal complementary target DNA. After the second round invader assay, a linear signal 20 amplification of greater than 10^6 signal/target/hr is obtained.

The present invention further provides a novel read-out method for improving the use of mobile solid support-based read-out technologies for detection of nucleic acid polymorphisms in a target nucleic acid utilizing a target oligonucleotide having a first complementarity region complementary to the target nucleic acid and a second

complementarity region, 5' to the first complementarity region, complementary to a capture oligonucleotide, which capture oligonucleotide is linked to a mobile solid support. The improved method can be applied to any of several methods of identifying a nucleic acid polymorphism, such as oligonucleotide ligation assay (OLA) or single base chain extension (SBCE), as described herein. The methods can be utilized to detect SNPs in genomic DNA as well as amplified DNA, RNA, etc., thus making it useful for a variety of purposes, including genotyping (such as for disease mutation detection and for parentage determinations in humans and other animals), pathogen detection and identification, and differential gene expression.

10 The present invention further provides the development of a simple method for multiplexing short sequencing reads (about 16 bases) in the same lane. One application to which this method can be applied is high-throughput yeast two-hybrid analysis. In this analysis, it is desired to sequence short regions of the interacting proteins, and then use a large database to determine the hit identification. Because 15 each bait analyzed generates approximately 100 hits, the present method to increase the efficiency of analysis was needed and therefore developed.

20 The invention can be utilized to analyze a nucleic acid sample that comprises genomic DNA, amplified DNA, such as a PCR product, cDNA, cRNA, a restriction fragment or any other desired nucleic acid sample. When one performs one of the herein described methods on genomic DNA, typically the genomic DNA will be treated in a manner to reduce viscosity of the DNA and allow better contact of a primer or probe with the target region of the genomic DNA. Such reduction in viscosity can be achieved by any desired method, which are known to the skilled artisan, such as DNase treatment or shearing of the genomic DNA, preferably lightly.

Amplified DNA can be obtained by any of several known methods. Sources of genomic DNA are numerous and depend upon the purpose of performing the methods, but include any tissue, organ or cell of choice. Oligonucleotides can be generated by amplification or by *de novo* synthesis, for example. Complementary 5 nucleic acids, *i.e.*, cRNA (obtained from a process wherein DNA is primed with a T7- RNA polymerase/specific sequence primer fusion, then T7 RNA polymerase is added to amplify the first strand to create cRNA) and cDNA, can be obtained by standard methods known in the art.

Thus, in the present methods, “nucleic acid” includes any of, for example, an 10 oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule. Often a primer or a probe in an example is an oligonucleotide, but the source of the primers or probes is not so limited herein.

15 As used in the claims, “a” and “an” can mean one or more, depending upon the context in which it is used.

In the basic SBCE method, a single oligonucleotide is attached to a detectably 20 tagged, mobile solid support, such as a bead or a rod, preferably that can be processed for detection of the tag quickly once the desired reaction has taken place, such as by a FACS-type system. For example, if one will ultimately fix the support in place prior to detection, a “tentagel” (“octopus”) can be used, then fixed in place prior to detection. Any desired tag can be utilized, such as a fluorescent tag, a radiolabel, or a magnetic tag. Other detection systems can be used, preferably, however, wherein the mobile solid support is passed over a detection device, such as a laser detection

device, capable of detecting and discerning the selected tags and labels (see, e.g., PCT publication WO 9714028). Detection systems can also be utilized wherein the mobile solid support, after performing any reactions, is fixed onto a two-dimensional surface and a detection device, such as a laser detection device, is passed over the fixed 5 mobile solid support. The mobile solid support can comprise any useful material, such as polystyrene-divinylbenzene. Detection of the mobile solid support and any nucleic acid or nucleotide associated with it, can be performed by FACS-based method, such as the Luminex FlowMetrix™ system.

In a typical assay, the oligonucleotide is designed such that the 5' end is 10 coupled to the bead. The 3' base ends at a nucleotide chosen relative to the polymorphic base, depending upon the assay being performed. For example, the 3' base of this primer or probe can end at the nucleotide 5' to the polymorphic base, it can end with a base corresponding to the polymorphic base. The length of the oligo in the SBCE method is not critical, but it does need to be long enough to support 15 hybridization by a nucleic acid sample, such as a PCR product generated from a region surrounding the SNP. Depending upon the assay to be performed, the primer or probe can be designed wherein an exact match is required or it may be designed to allow some mismatch upon initial hybridization to the sample nucleic acid.

In a typical assay, a nucleotide capable of chain termination is utilized. Such 20 chain termination is a termination event that occurs before the same labeled base occurs again in the target sequence. Such nucleotides are known in the art and include, for example, a dideoxynucleotide (when polymerase is used in the extension reaction), a thiol derivative (when polymerase is used in the extension reaction), a 3' deoxynucleotide (using reverse transcriptase in the extension reaction), or a 3'

deoxyribonucleotide (using reverse transcriptase in the extension reaction). Any of these nucleotides can be, for example, a dinucleotide, a trinucleotide, or a longer nucleic acid. Thus, one can have, for example, a bank of dinucleotides or longer nucleic acids such that within the bank one has optional nucleotides at more than one 5 location.

Thus, in the present method, the labeling step is typically performed in solution (thus providing efficient hybridization), and the analysis step can be performed either in solution or on a solid, non-mobile support.

The present invention therefore provides a method of identifying a selected 10 nucleotide in a first nucleic acid comprising

- (a) contacting the first nucleic acid with a nucleic acid primer linked at its 5' end to a detectably tagged mobile solid support wherein the nucleic acid primer comprises a region complementary to a section of the first nucleic acid that is directly 3' of and adjacent to the selected nucleotide, under hybridization conditions that allow 15 the first nucleic acid and the nucleic acid primer to form a hybridization product;
- (b) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension; and
- (c) detecting the presence or absence of a label incorporated into the hybridization 20 product, the presence of a label indicating the incorporation of the labeled nucleotide into the hybridization product, and the identity of the incorporated labeled nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the first nucleic acid.

In a specific embodiment, a primer is designed such that its 3' base ends at the nucleotide immediately 5' of the polymorphic base. A set of 4 dideoxynucleotide triphosphate mixtures are generated. Each mixture contains one of four labeled dideoxynucleotide molecules that have been chemically-coupled to a fluorescein molecule (i.e., ddATP-F, ddCTP-F, ddGTP-F or ddTTP-F), and three non-labeled dideoxynucleotide triphosphates. In one format, the PCR product is added to the bead and the bead aliquoted into 2 or more tubes. The chain-terminating mixtures are dispensed to the tubes and a polymerase is added to generate the SBCE reaction tubes. The polymerase will extend a base onto the 3' end of the bead-attached oligo, this base being the complement of the base at the polymorphic site. The reaction tubes are analyzed by FlowMetrix and the appearance of a label in a particular reaction tube on a particular bead will indicate the polymorphic base at the site.

A comparison of the present method with a hybridization method is illustrative of the utility of the present invention. In the SNP analysis by hybridization, 2 oligos on 2 beads in the same tube are used to generate the material to be read for analysis. In the SCBE method, the same oligo on the same bead is analyzed in 2 tubes with 2 different labeled dideoxynucleotides. Although the method has been exemplified herein using fluorescein as the dye read-out, one can couple this method with biotinylated or other appropriately-modified nucleotides.

The present methods can be performed wherein the chain terminating nucleotide is a dideoxynucleotide and the primer extension is performed in the presence of one labeled, identified dideoxynucleotide and three different, non-labeled dideoxynucleotides. In another embodiment, the chain terminating nucleotide is a dideoxynucleotide, wherein the primer extension is performed in the presence of a

first identified dideoxynucleotide labeled with a first detectable label, a second identified dideoxynucleotide labeled with a second detectable label, a third identified dideoxynucleotide labeled with a third detectable label and a fourth identified dideoxynucleotide labeled with a fourth detectable label, and wherein detection of the 5 presence of the first, the second, the third or the fourth detectable label in the hybridization product indicates the identity of the nucleotide complementary to the selected nucleotide as the first, the second, the third or the fourth dideoxynucleotide, respectively.

Because it is possible to thermal cycle the FlowMetrix beads (Ralph McDade, 10 pers. communication), one can perform a genomic scan using the SBCE method. In this method, the genomic DNA could be sheared, or treated with DNase to reduce viscosity, and cycled against oligos attached to the beads. Because of the vastly greater complexity of the template DNA, it may necessitate the need for extended hybridization optimization and cycling times. Since one would be essentially 15 performing a Cot analysis on the beads. Use of these beads and SBCE for SNP identification and DNA sequencing should be apparent from the above description.

Thus, the present invention provides a method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising 20 (a) performing an amplification of the genomic DNA using a first nucleic acid primer comprising a region complementary to a section of one strand of the nucleic acid that is 5' of the selected nucleotide, and a second nucleic acid primer complimentary to a section of the opposite strand of the nucleic acid downstream of the selected nucleotide, under conditions for specific amplification of the region of the selected nucleotide between the two primers, to form a PCR product;

(b) contacting the PCR product with a first nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the first nucleic acid comprises a region complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions to form a hybridization product;

5 (c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

(d) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

10 (e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

15 The PCR product can be in single-stranded form.

The present invention further provides a method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

20 (a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of the nucleic acid that is directly 5' of the selected nucleotide, and using T7 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the

second strand complementary to the cRNA strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;

- (b) contacting the amplification product with a first oligonucleotide linked at its 5' end to a detectably tagged mobile solid support under hybridization conditions to form a hybridization product;
- (c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- 10 (d) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- 15 (e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

The labeled chain -terminating nucleotide can be, for example, a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. The 20 amplification product can be in single-stranded form.

Furthermore, one can design and synthesize some primers to sit just downstream of the nucleic acids attached to the beads. These can be the primers used to i) make the first strand cDNA, and, ii) with a set that has attached to it the T7 RNA polymerase, can be used to make cRNA. To make the second strand, if needed for the

cRNA, one can use a second primer set that sits outside of the sequence attached to the beads, but just upstream of it. By having the primers off the bead-oligo, they shouldn't interfere by binding. The primers can be made FITC-labeled for the cDNA.

The present method further provides a method of determining a selected 5 nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising (a) contacting the genomic DNA with a first primer linked at its 5' end to a detectably tagged mobile solid support, wherein the first primer comprises a first region complementary to a section of one strand of the genomic DNA that is directly 5' of and adjacent to the selected nucleotide under hybridization conditions for 10 forming a specific hybridization product;

(b) performing a primer extension reaction with the specific hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

(c) detecting the presence or absence of a label incorporated into the hybridization 15 product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

(d) comparing the identity of the selected nucleotide with a non-polymorphic 20 nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

The DNA can be in single-stranded form. The labeled chain-terminating nucleotide can be, for example, a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. In such a method, the hybridization time should be

of a length sufficient to allow hybridization of the first primer to the genomic DNA since the genomic DNA has not been amplified in this specific embodiment. Thus relatively long hybridization times may be utilized, such as, for example, 12 hours, 24 hours, 48 hours, as is known in the art for hybridization to genomic DNA (see, e.g.,

5 Sambrook, *et al. Molecular Cloning: A Laboratory Manual*, 2d ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989).

For any of the herein described reactions, alternative polymerases can be employed, such as a polymerase with a temperature condition for function, or a polymerase with a particular specificity for nucleotides, such as a polymerase that 10 preferentially incorporates dideoxynucleotides (see, e.g., Sambrook, *et al.*). The skilled artisan is familiar with such polymerases, and new polymerases, as they are discovered, can be incorporated into the methods, given the teachings herein..

The present invention additionally provides the use of the beads in an oligonucleotide-ligation assay (OLA) format, i.e., in which one can hybridize 15 genomic DNA, cRNA or PCR product to a first nucleic acid attached to the bead, then come in with a second nucleic acid with a fluorescent label, then add ligase, and wherein the second nucleic acid has at its 3' end the polymorphic bases. Thus, the present invention provides a method of identifying a selected nucleotide in a first nucleic acid comprising
20 (a) contacting the first nucleic acid with (i) a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of the selected nucleotide and wherein the second nucleic acid terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and (ii) a third,

fluorescently labeled nucleic acid, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,

under hybridization conditions that allow the first nucleic acid and the second 5 nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product;

(b) adding to the hybridization product a ligase under ligation conditions; and

(c) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the nucleic acid linked to the mobile solid support, 10 the presence of the label indicating the ligation of the labeled third nucleic acid to the second nucleic acid linked to the mobile solid support, and the identity of the test nucleotide in the second nucleic acid indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

This oligonucleotide ligation assay can be performed both (a) wherein the 15 polymorphic base is located at the 5' side of either the reporter or acceptor oligonucleotide, or (b) wherein the polymorphic base is located at the 3' side of either the reporter or acceptor oligonucleotide.

The first nucleic acid can be genomic DNA (treated to reduce viscosity, e.g., by 20 DNase treatment or by shearing), amplified nucleic acid such as a PCR product, an oligonucleotide, a 16s ribosomal RNA, a DNA fragment, an RNA molecule, a cDNA molecule, a cRNA molecule, restriction enzyme-generated DNA fragment, size-selected DNA, Bridge-amplified DNA, 16S RNA, 16S DNA or any other desired nucleic acid.. Any selected ligase can be used, such as T4 DNA ligase. A

thermostable ligase would be particularly useful. *See, generally* Wu and Wallace, *Genomics* 4: 560-569 (1989).

The present invention additionally encompasses the use in the OLA readout of degenerate reporter oligonucleotides, preferably the use of 8-mer oligonucleotides 5 wherein 6 of the bases are chosen to be specific to the target nucleic acid and 2 of the bases are variable, or wobble or degenerate, positions. The degeneracy can be placed in any position in the reporter oligonucleotide; however, preferable positions can be positions 3, 4, 5, and 6. Preferable variable position combinations in a selected oligonucleotide can be positions 3 and 6, positions 4 and 5, and positions 3 and 4.

10 Thus, one can synthesize all possible "6+2-mers" as reagents for use in an assay, whereas synthesis of all possible 8-mers is not practicable. Furthermore, non-natural derivatives, such as inosine, can be utilized in the reporter oligonucleotides. For example, the present invention includes an OLA readout wherein the reporter oligonucleotide is an 8-base complementary 8-mer conjugated to a reporter molecule 15 or hapten to which a reporter molecule can be conjugated by means of a hapten-recognizing intermediary (e.g., antibody, avidin, streptavidin). The present invention further includes an OLA readout wherein the reporter oligonucleotide is an 6-base complementary 8-mer ("6+2-mer") conjugated to a reporter molecule or hapten to which a reporter molecule can be conjugated by means of a hapten-recognizing 20 intermediary. The two non-complementary bases can be any of the four natural bases or can be a non-natural derivative capable of forming a non-helix disturbing duplex structure. The non-complementary bases can preferably be located at positions 3 and 6 or positions 4 and 5. Non-natural base derivatives and/or 6+2-mers can be components of a kit for use in performing the detection methods described herein.

Further, one can employ a 'Taqman' approach wherein one incorporates Dye quenchers and Dye acceptors into the attached oligos and asks for the polymerase to remove the dye quencher in a repair reaction.

The invention further employs hybridization methods wherein two 5 nucleic acids are hybridized to the sample nucleic acid but the step of ligation can be omitted and a match instead detected by fluorescence energy transfer between the two nucleic acids hybridized to the sample nucleic acid. The two hybridizing nucleic acids are designed such that the 3' end of the nucleic acid linked to the bead is a test base, and when it is complementary to the polymorphic base, and a single wavelength 10 of light is directed onto the sample, one can detect a transfer of energy, read as a second wavelength of light. A second reader can be employed for this detection of this second wavelength. Thus, the present invention provides a method of identifying 15 a selected nucleotide in a first nucleic acid comprising

- (a) contacting the first nucleic acid with
 - 15 (i) a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support and linked at its 3' end to a fluorescent label, wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of the selected nucleotide and wherein the second nucleic acid terminates at its 3' end in a test 20 nucleotide positioned to base-pair with the selected nucleotide, and
 - (ii) a third nucleic acid fluorescently labeled at its 5' end, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,

under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product; and

(c) detecting the presence or absence of fluorescent energy transfer between
5 the fluorescent label at the 3' end of the second nucleic acid and the fluorescent label
at the 5' end of the third nucleic acid, the presence of fluorescent energy transfer
indicating the hybridization of the test nucleotide to the first nucleic acid, and the
identity of the hybridized test nucleotide in the second nucleic acid indicating the
identity of the nucleotide complementary to the selected nucleotide, thus identifying
10 the selected nucleotide. The detection of the fluorescence energy transfer (FET) can
be performed after dissociation of the hybridized nucleic acids.

The present invention also provides a method for determining the sequence of a polymorphic base comprising: a first nucleic acid attached at a 5' end to a mobile solid support and having a 3' end adjacent to a polymorphic base on a second nucleic acid;
15 a third nucleic acid with an attached reporter moiety that is complementary to a region adjacent to the polymorphic base of the second nucleic acid; the first nucleic acid and the third nucleic acid together defining a gap opposite the polymorphic base; a nucleotide that is complementary to one of a set of two possible polymorphic bases, a polymerase, and a ligase; wherein the polymerase is able to polymerize the nucleotide
20 across the gap if the nucleotide is complementary to the polymorphic base; the ligase is able to ligate the newly polymerized nucleotide to the reporter-attached third nucleic acid; and a means for detecting the reporter covalently linked to the bead. Specifically, the present invention provides a method of identifying a selected nucleotide in a first nucleic acid comprising

(a) contacting the first nucleic acid with

- (i) a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
- (ii) a third nucleic acid fluorescently labeled, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product, wherein the first, second and third nucleic acids form a hybridization product that defines a gap opposite the selected nucleotide;

(b) adding a test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation; and

(c) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the nucleic acid linked to the mobile solid support, the presence of the label indicating the polymerization of the test nucleic acid to the second nucleic acid and ligation of the labeled third nucleic acid to the second nucleic acid linked to the mobile solid support, and the identity of the test nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

The polymerase can preferably be a non-strand displacing polymerase. Further, it can be a thermostable polymerase. The ligase can be a DNA ligase. Further, it can be a thermostable ligase.

The present invention further provides a method of detecting a single base polymorphism comprising using an enzyme or chemical to modify or endonucleolytically cleave a mismatched base at the polymorphic site in a nucleic acid, resulting in the loss of an attached reporter or in a modification, and detecting a loss of the reporter or detecting the modification, thus resulting in a labeling means for the identification of the modification. In one example, an end-labeled (such as 5 with FITC) genomic fragment or a labeled (such as with FITC) PCR fragment is hybridized to an oligonucleotide and attached to a bead, then the construct is treated with an enzyme that recognizes and/or restricts mispaired DNA (such as FITC-labeled recA, mutS or T7 enzyme) and analyzed for the addition or loss of the label. In another example, a chemical recognizing single stranded regions of DNA and capable 10 of modifying the region is utilized, and the modification is detected.

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Furthermore, any of the herein described methods can be utilized in a method for quantitating expression of a selected nucleic acid in a sample. Thus, it can be used, for example, for differential gene expression wherein the expression of a selected gene is quantitated and compared to a standard or some other reference. For 20 this method, a gene fragment from a region of interest or a region that distinguishes the gene (or allele or haplotype or polymorphism) of interest is linked at its 5' end to a detectably labeled mobile solid support; message (e.g., RNA, cDNA, cRNA) is hybridized to the fragment, and fluorescence is quantitated by performing a primer extension reaction, a ligase reaction or a hybridization/fluorescence energy transfer

reaction, such as that described herein. The nucleic acid probe can comprise a region complementary to a section of the selected nucleic acid unique to the nucleic acid. A standard, such as that from a normal subject, or a diseased/ afflicted subject, or a particular tissue or organ, or a particular species, can be used as a comparison 5 reference to draw conclusion regarding the quantity detected in the sample.

Specifically, the present invention provides a method of detecting a result from an identification reaction to identify a selected nucleotide in a target nucleic acid comprising:

- a) contacting a target oligonucleotide comprising a first complementarity 10 region and a second complementarity region, wherein the second complementarity region is 5' of the first complementarity region and wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide, with a sample comprising the 15 target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a first hybridization product;
- b) performing a selected identification reaction with the first hybridization 20 product to determine the identity of the selected nucleotide wherein a selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;

- c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form a second hybridization product; and
- d) detecting and/or identifying the label of the labeled detection product in the second hybridization product,
the presence and or identity of the label indicating the identity of the selected 10 nucleotide in the target nucleic acid.

The basic method thus involves the use of a capture oligonucleotide, linked to a mobile solid support (such as a bead), to isolate a reaction product from a reaction. To facilitate this isolation, a "target oligonucleotide" is designed which comprises, in addition to a first complementarity region, which is a region complementary to a 15 region of the target nucleic acid, a second complementarity region, which is located 5' of the first complementarity region, and which is complementary to the nucleotide sequence of the capture oligonucleotide. Thus, before or after a reaction (such as SBCE or OLA), the capture oligonucleotide can be utilized in a hybridization reaction to isolate the target oligonucleotide in its reacted form (e.g., as a ligation product or as 20 a primer extension product). Thus, one is not obligated, as in many other assays, to synthesize a bead specifically for each oligonucleotide (e.g., the "first complementarity region of the target oligonucleotide in the present invention) that is to be hybridized to the target nucleic acid.

The present invention additionally encompasses the use in the OLA readout of degenerate reporter oligonucleotides, preferably the use of 6+2-mers as described herein. Such reporter oligonucleotides can be a component of a useful kit for performing the detection methods herein.

5 The capture oligonucleotide can be designed such that it does not specifically hybridize, *i.e.*, is not sufficiently complementary for specific hybridization to occur, to the target nucleic acid. For example, it can include nucleotide usage not typically found in the target species (such as human). If the target sequence is fully known, the capture sequence can be selected as a sequence which does not occur in the target 10 sequence. A capture oligonucleotide can be of any desired length so long as it is sufficiently long so as to selectively hybridize to a first complementarity region of a target oligonucleotide (under selective hybridization conditions, *e.g.*, stringent hybridization conditions, as known to one skilled in the art), and not so long as to interfere with either the identification reaction being performed with the target 15 oligonucleotide or the hybridization reaction between the capture oligonucleotide and the target oligonucleotide. The capture oligonucleotide length selected can also be a function of how many different capture oligonucleotides one desires to use in any selected use. For example, the capture oligonucleotide can be 8, 10, 12, 15, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40 or more nucleotides. A preferred length is 20 around 25 nucleotides, such as 23, 24, 25, 26, 27 or 28 nucleotides. However, other oligonucleotide lengths can be utilized. Optimal length for any specific use can be determined according to the specific nucleic acid composition, as will be known to those skilled in the art.

One can advantageously create a bank of several capture oligonucleotides, each linked to a different color of bead. A bank of complementary regions can be maintained for use in generating target oligonucleotides for any specific target nucleic acid. Thus, one can utilize a defined set of beads, and simply create new target 5 nucleotides as necessary for any given detection task.

The present invention provides a method of detecting a reaction product to identify a selected nucleotide in a target nucleic acid comprising:

- a) contacting a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first 10 complementarity region comprises the oligonucleotide primer and the second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and wherein the oligonucleotide primer comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected 15 nucleotide, with a sample comprising the target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a 20 first hybridization product;
- b) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;

c) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

5 detecting the presence or absence of a label in the isolated second hybridization product, the presence of a label indicating the incorporation of the labeled nucleotide into the primer extension product, and the identity of the identified

10 incorporated labeled nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

In a typical assay, the target oligonucleotide is designed such that the 5' end comprises second complementarity region and later allows for hybridization to a

15 complementary capture oligonucleotide linked to a mobile solid support, and the 3' end comprises a first complementarity region complementary a region of the target nucleic acid just 3' of the polymorphic base. The 3' base ends at a nucleotide chosen relative to the polymorphic base, depending upon the assay being performed. For example, the 3' base of this target oligonucleotide can end at the nucleotide 5' to the

20 polymorphic base, or it can end with a base corresponding to the polymorphic base. The present invention additionally provides the use of the beads in an oligonucleotide-ligation assay (OLA) format, i.e., in which one can hybridize genomic DNA, cRNA or PCR product to a target oligonucleotide having a first complementarity region that is complementary to a section of the target nucleic acid that is directly 3' of the

selected nucleotide, then come in with a reporter oligonucleotide having a fluorescent label, then add ligase, and wherein the target oligonucleotide has at its 3' end the polymorphic bases. For a typical OLA reaction with capture read out, the reagents can comprise: a target oligonucleotide containing two regions of complementarity; a 5 first complementarity region of the target oligo is complementary to a region immediately adjacent to a single nucleotide polymorphism to be analyzed, a second complementarity region of the target oligonucleotide which is complementary to a capture oligonucleotide; a capture oligonucleotide that is covalently coupled to a mobile solid support; a reporter oligonucleotide complementary to the the region overlapping the SNP and containing a means for readout, and a 3' base on the strand 10 opposite the SNP position; a ligase capable of ligating the reporter and the target if the base on the reporter that is opposite the SNP is complementary. In one embodiment of the method, the ligation reaction is then added to the capture-oligonucleotide-coupled mobile solid support and hybridization of the second complementarity region 15 to the bead is allowed to occur under standard hybridization conditions. Readout of the reporter could be performed using a Luminex LX100-type machine.

The advantages to this system include the reduced number of bead sets needed to analyze many different SNPs, i.e., if given 100 bead colors, then one could synthesize only 100 capture oligonucleotides and use them over and over again in the 20 different wells.

Thus, the present invention provides a method of detecting a result from an identification reaction (OLA) to identify a selected nucleotide in a target nucleic acid comprising:

- a) hybridizing (i) a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide and the second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and (ii) a fluorescently labeled reporter oligonucleotide comprising a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected nucleotide, to a sample comprising the target nucleic acid, under hybridization conditions that allow specific hybridization between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide and that also allow specific hybridization between the reporter oligonucleotide and the section of the target nucleic acid complementary to the reporter oligonucleotide, to form a first hybridization product that defines a gap opposite the selected nucleotide;
- b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation to form a labeled product;
- c) dissociating the hybridized nucleic acids;
- d) isolating the labeled product by contacting the labeled product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence

complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form a second hybridization product; and

e) detecting the presence or absence of the label in the second hybridization product,

the presence of the label indicating polymerization of the identified test

nucleotide to the target oligonucleotide and ligation of the labeled reporter

oligonucleotide to the polymerized target oligonucleotide, and the identity of

the identified test nucleotide indicating the identity of the nucleotide

complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

The target nucleic acid can be genomic DNA treated to reduce viscosity, an oligonucleotide, a 16s ribosomal RNA, a 16S DNA, a PCR product, a DNA fragment, a restriction enzyme-generated DNA fragment, size-selected DNA, Bridge-amplified DNA, an RNA molecule, a cDNA molecule or a cRNA molecule.

The present invention further provides a method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

a) performing an amplification of the genomic DNA using a first nucleic acid primer comprising a region complementary to a section of one strand of the nucleic acid that is 5' of the selected nucleotide, and a second nucleic acid primer complimentary to a section of the opposite strand of the nucleic acid downstream of the selected nucleotide, under conditions for specific amplification of the region of the selected nucleotide between the two primers, to form a PCR product;

- b) contacting the PCR product with a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected 5 nucleotide, under hybridization conditions to form a first hybridization product;
- c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;
- d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form 10 an isolated second hybridization product;
- e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the 15 selected nucleotide; and
- f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the 20

non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

The present invention further provides a method of determining a selected 5 nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of the nucleic acid that is directly 5' of the selected nucleotide, and using T7 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the second strand complementary to the cRNA strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;
- b) contacting the amplification product with a target oligonucleotide 10 comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions to 15 form a first hybridization product;
- c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under 20 conditions for primer extension to form a primer extension product;

d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;

5 e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

10 f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

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The labeled chain -terminating nucleotide can be, for example, a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. The amplification product can be in single-stranded form. Furthermore, one can design 20 and synthesize some primers to sit just downstream of the target oligonucleotides.

The present method further provides a method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

a) contacting the genomic DNA with a target oligonucleotide comprising a first complementarity region and a second complementarity region,

wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions for 5 forming a specific first hybridization product;

b) performing a primer extension reaction with the specific first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

c) isolating the primer extension product by contacting the primer extension 10 product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;

d) detecting the presence or absence of a label incorporated into the second 15 hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

The DNA can be in single-stranded form. The labeled chain -terminating nucleotide can be, for example, a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. In such a method, the hybridization time should be of a length sufficient to allow hybridization of the first primer to the genomic DNA 5 since the genomic DNA has not been amplified in this specific embodiment. Thus relatively long hybridization times may be utilized, such as, for example, 12 hours, 24 hours, 48 hours, as is known in the art for hybridization to genomic DNA (*see, e.g.,* Sambrook, *et al.*).

In reactions utilizing a ligase, any selected ligase can be used, such as T4 DNA 10 ligase. A thermostable ligase would be particularly useful. *See, generally* Wu and Wallace, *Genomics* 4: 560-569 (1989).

The invention further employs hybridization methods wherein two nucleic acids are hybridized to the sample nucleic acid but the step of ligation can be omitted and a match instead detected by fluorescence energy transfer between the two nucleic 15 acids hybridized to the sample nucleic acid. The two hybridizing nucleic acids are designed such that the 3' end of the target oligonucleotide is a test base, and when it is complementary to the polymorphic base, and a single wavelength of light is directed onto the sample, one can detect a transfer of energy, read as a second wavelength of light. A second reader can be employed for this detection of this second wavelength. 20 Thus, the present invention provides a method of identifying a selected nucleotide in a target nucleic acid comprising

a) contacting the target nucleic acid with

i. a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity

region is complementary to a section of the first nucleic acid that is directly 5' of the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in an identified test nucleotide positioned to base-pair with the selected nucleotide, and wherein the second

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complementarity region is 5' to the first complementarity region, and

- ii. a fluorescently labeled reporter oligonucleotide, wherein the reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide,

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under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to hybridize and the target nucleic acid and the reporter oligonucleotide to hybridize, thus forming a first hybridization product;

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- b) adding to the first hybridization product a ligase under ligation conditions;
- c) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization

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conditions to form an isolated second hybridization product; and detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product, the presence of the label indicating the ligation of the labeled reporter oligonucleotide to the target oligonucleotide, and the identity of the test nucleotide in the target oligonucleotide

indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide. The detection of the fluorescence energy transfer can be performed after dissociation of the hybridized nucleic acids.

The present invention additionally provides a method of identifying a selected 5 nucleotide in a target nucleic acid comprising

- a) contacting the target nucleic acid with
 - i. a target oligonucleotide linked at its 3' end to a fluorescent label, wherein the target oligonucleotide comprises a first complementarity region that is complementary to a section of the target nucleic acid that is directly 3' of 10 the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and wherein the target oligonucleotide has a second complementarity region 5' of the first complementarity region, and
 - ii. a reporter oligonucleotide fluorescently labeled at its 5' end, wherein the reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected 15 nucleotide,
- under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to hybridize and the target nucleic acid and the reporter oligonucleotide to hybridize, to form a first hybridization product;
- b) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target

oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

detecting the presence or absence of fluorescent energy transfer between the fluorescent label at the 3' end of the target oligonucleotide and the fluorescent label at 5 the 5' end of the reporter oligonucleotide in the second hybridization product, the presence of fluorescent energy transfer indicating the hybridization of the identified test nucleotide to the target nucleic acid, and the identity of the hybridized test nucleotide in the target oligonucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

10 The present invention also provides a method for determining the sequence of a polymorphic base in a target nucleic acid which can utilize a kit comprising one or more of the following: a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region is complementary to a section of the target nucleic acid having a 3' end adjacent to and directly 5' of the polymorphic base on the target nucleic acid; a reporter oligonucleotide with an attached reporter moiety that is complementary to a region immediately adjacent to and 3' of the polymorphic base of the target nucleic acid; the target oligonucleotide and the reporter oligonucleotide together defining a gap 15

15 complementary to the polymorphic base; a capture oligonucleotide that is covalently linked to a mobile solid support (such as a polystyrene-divinylbenzene bead), wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide; a nucleotide that is complementary to one of a set of two possible polymorphic bases; a polymerase, and

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a ligase, wherein the polymerase is able to polymerize the nucleotide across the gap if the nucleotide is complementary to the polymorphic base and wherein the ligase is able to ligate the newly polymerized nucleotide to the reporter oligonucleotide; and a means for detecting the reporter covalently linked to the head. Further, the present

5 invention provides a method of identifying a selected nucleotide in a target nucleic acid comprising

- a) contacting the target nucleic acid with
 - i. a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first
 - 10 complementarity region, wherein the first complementarity region is complementary to a section of the target nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
 - ii. a reporter oligonucleotide fluorescently labeled, wherein the reporter oligonucleotide comprises a region complementary to a section of the
 - 15 second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,
- under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to form a hybridization product and the target nucleic acid and the reporter oligonucleotide to form a hybridization product, wherein the target nucleic acid, target oligonucleotide and reporter oligonucleotide form a hybridization product that defines a gap opposite the selected nucleotide;
- b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation;

- c) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and
- d) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product,

the presence of the label indicating the polymerization of the test nucleic acid to the target oligonucleotide and ligation of the labeled reporter oligonucleotide to the target oligonucleotide linked to the mobile solid support, and the identity of the test nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

The polymerase can preferably be a non-strand displacing polymerase.

Further, it can be a thermostable polymerase. The ligase can be a DNA ligase.

Further, it can be a thermostable ligase.

Furthermore, any of the herein described methods can be utilized in a method for quantitating expression of a selected nucleic acid in a sample. Thus, it can be used, for example, for differential gene expression wherein the expression of a selected gene is quantitated and compared to a standard or some other reference. For this method, a gene fragment from a region of interest or a region that distinguishes the gene (or allele or haplotype or polymorphism) of interest is selected for use as the first complementarity region of a target oligonucleotide; message (e.g., RNA, cDNA, cRNA) is hybridized to the target oligonucleotide, and fluorescence is quantitated by

performing a primer extension reaction, a ligase reaction or a hybridization/fluorescence energy transfer reaction, such as that described herein. A corresponding capture oligonucleotide (complementary to a second complementarity region utilized in the target oligonucleotide) linked to a mobile solid support is 5 utilized to capture the reaction product. The first complementarity region of a target oligonucleotide can comprise a region complementary to a section of the selected nucleic acid unique to the nucleic acid. A standard, such as that from a normal subject, or a diseased/ afflicted subject, or a particular tissue or organ, or a particular species, can be used as a comparison reference to draw conclusions regarding the 10 quantity detected in the sample.

Thus the present invention provides a method of quantitating expression of a selected nucleic acid in a sample comprising

- a) contacting (i) message nucleic acid isolated from a selected source with (ii) a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region comprises a region complementary to a section of the selected nucleic acid;
- 15 b) performing a selected identification reaction with the first hybridization product to determine the identity of the selected nucleotide wherein a selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;
- 20 c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence

complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated hybridization product; and

5 d) quantitating the fluorescence in the isolated hybridization product, the quantity of fluorescence indicating the quantity of the selected nucleic acid in the sample.

For any of these methods described herein, a sample can be, for example, any body sample that contains message, such as organ tissue and/or cells, such as blood, red or white blood cells, bone marrow, liver, kidney, brain, skin, heart, lung, spleen, 10 pancreas, gall bladder, muscle, neuronal cells, neurons, precursor cells, ovaries, testicles, uterus, glands.

Additionally provided are kits for detecting a single base polymorphism, wherein a kit comprises a detectably tagged mobile solid support, such as a polystyrene-divinylbenzene bead, and one to four modified (chain-terminating) 15 nucleotide(s), such as a 3' deoxynucleotide, a 3' deoxyribonucleotide, a thiol derivative, or a dideoxynucleotide. The kit can additionally comprise a polymerase, and in particular, a polymerase that preferentially incorporates the modified nucleotide. The kit can additionally comprise a ligase. The kit can also comprise one or more fluorescent label for labeling the nucleic acid(s). For genomic DNA uses, the 20 kit can further comprises a DNase for reducing the viscosity of the DNA. The kit can further contain an array of combinations of dinucleotides and/or a collection of combinations of trinucleotides.

The following documents provide information regarding various technologies:

PCT publication WO 9714028 (Luminex Corp.).

Australian patent AU 9723205 (based on WO 9735033 (97/09/25)) (Molecular Tool Inc.)

European patent publication EP 754240 (based on WO 9521271) (Molecular Tool Inc.)

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European patent publication EP 736107 (based on WO 9517524) (Molecular Tool Inc.)

U.S. Pat. No. 5,610,287 (97/03/11) (Molecular Tool Inc.)

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European patent publication EP 726905 (based on WO 9512607) (Molecular Tool Inc.)

U.S. Pat. No. 5,518,900 (94/07/21) (Molecular Tool Inc.)

European patent publication EP 576558 (based on WO 9215712) (Molecular Tool Inc.)

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Throughout this application, various publications are referenced. These publications are hereby incorporated by reference in their entirety.

While the invention has been described with respect to certain specific embodiments, it will be appreciated that many modifications and changes may be made by those skilled in the art without departing from the spirit of the invention. It is intended, therefore, by the appended claims, to cover all such modification and 20 changes as fall within the true spirit and scope of the invention.

What is claimed is:

1. A method of identifying a selected nucleotide in a first nucleic acid comprising
 - a) contacting the first nucleic acid with a nucleic acid primer linked at its 5' end to a detectably tagged mobile solid support wherein the nucleic acid primer comprises a region complementary to a section of the first nucleic acid that is directly 3' of and adjacent to the selected nucleotide, under hybridization conditions that allow the first nucleic acid and the nucleic acid primer to form a hybridization product;
 - b) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension; and
 - c) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled nucleotide into the hybridization product, and the identity of the incorporated labeled nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the first nucleic acid.
- 20 2. The method of claim 1, wherein the first nucleic acid is an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.

3. The method of claim 1, wherein the labeled chain -terminating nucleotide is a 3'deoxyribonucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxyribonucleotide.
4. The method of claim 1, wherein the chain terminating nucleotide is a 5 dideoxyribonucleotide and the primer extension is performed in the presence of one labeled, identified dideoxyribonucleotide and three different, non-labeled dideoxyribonucleotides.
5. The method of claim 1, wherein the chain terminating nucleotide is a 10 dideoxyribonucleotide, wherein the primer extension is performed in the presence of a first identified dideoxyribonucleotide labeled with a first detectable label, a second identified dideoxyribonucleotide labeled with a second detectable label, a third identified dideoxyribonucleotide labeled with a third detectable label and a fourth identified dideoxyribonucleotide labeled with a fourth detectable label, and wherein detection of the presence of the first, the second, the third or the fourth detectable 15 label in the hybridization product indicates the identity of the nucleotide complementary to the selected nucleotide as the first, the second, the third or the fourth dideoxyribonucleotide, respectively.
6. The method of claim 1, wherein the mobile solid support is a bead.
7. The method of claim 4, wherein the bead is a polystyrene-divinylbenzene.
- 20 8. The method of claim 1, wherein the mobile solid support is detectably tagged with dye, a radiolabel, or a magnetic tag.
9. The method of claim 1, wherein the first nucleic acid is an amplification product.
10. The method of claim 1, wherein the first nucleic acid is a PCR product.

11. The method of claim 1, wherein the detecting is performed by passing the mobile solid support over a laser detection device capable of detecting/distinguishing the detectable tag.

12. The method of claim 1, wherein the detecting is performed by fixing the mobile solid support onto a two-dimensional surface and passing a laser detection device capable of detecting/distinguishing the detectable tag over the solid support.

13. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

10 a) performing an amplification of the genomic DNA using a first nucleic acid primer comprising a region complementary to a section of one strand of the nucleic acid that is 5' of the selected nucleotide, and a second nucleic acid primer complimentary to a section of the opposite strand of the nucleic acid downstream of the selected nucleotide, under conditions for specific amplification of the region of the selected nucleotide between the 15 two primers, to form a PCR product;

20 b) contacting the PCR product with a first nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the first nucleic acid comprises a region complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions to form a hybridization product;

25 c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

- d) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

14. The method of claim 13, wherein the labeled chain -terminating nucleotide is a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

15. The method of claim 13, wherein the DNA has been sheared to reduce viscosity.

16. The method of claim 13, wherein the DNA has been treated with DNase to reduce viscosity.

17. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of the nucleic acid that is directly 5' of the selected nucleotide, and using T7 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the second strand complementary to the cRNA

strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;

5 b) contacting the amplification product with a first oligonucleotide linked at its 5' end to a detectably tagged mobile solid support under hybridization conditions to form a hybridization product;

c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

d) detecting the presence or absence of a label incorporated into the

10 hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

15 e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

18. The method of claim 17, wherein the labeled chain -terminating nucleotide is a

20 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

19. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- a) contacting the genomic DNA with a first primer linked at its 5' end to a detectably tagged mobile solid support, wherein the first primer comprises a first region complementary to a section of one strand of the genomic DNA that is directly 5' of and adjacent to the selected nucleotide under hybridization conditions for forming a specific hybridization product;
- b) performing a primer extension reaction with the specific hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- c) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- d) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

20. The method of claim 19, wherein the labeled chain-terminating nucleotide is a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

21. A method of identifying a selected nucleotide in a first nucleic acid comprising

- a) contacting the first nucleic acid with

- i. a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of the selected nucleotide and wherein the second nucleic acid terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and
- ii. a third, fluorescently labeled nucleic acid, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,

under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product;

- b) adding to the hybridization product a ligase under ligation conditions; and
- c) detecting the presence or absence of the fluorescent label, after 15 dissociation of the hybridized nucleic acids, in the nucleic acid linked to the mobile solid support, the presence of the label indicating the ligation of the labeled third nucleic acid to the second nucleic acid linked to the mobile solid support, and the identity of the test nucleotide in the second nucleic acid indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

20 22. The method of claim 21, wherein the first nucleic acid is genomic DNA treated to reduce viscosity, an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.

23. A method of identifying a selected nucleotide in a first nucleic acid comprising

- a) contacting the first nucleic acid with
 - i. a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support and linked at its 3' end to a fluorescent label, wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of the selected nucleotide and wherein the second nucleic acid terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and
 - ii. a third nucleic acid fluorescently labeled at its 5' end, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,
- under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product; and
- b) detecting the presence or absence of fluorescent energy transfer between the fluorescent label at the 3' end of the second nucleic acid and the fluorescent label at the 5' end of the third nucleic acid, the presence of fluorescent energy transfer indicating the hybridization of the test nucleotide to the first nucleic acid, and the identity of the hybridized test nucleotide in the second nucleic acid indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

24. A method of identifying a selected nucleotide in a first nucleic acid comprising

- a) contacting the first nucleic acid with

- i. a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
- 5 ii. a third nucleic acid fluorescently labeled, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,
under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product, wherein the first, second and third nucleic acids form a hybridization product that defines a gap opposite the selected nucleotide;
- 10 b) adding a test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation; and
- 15 c) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the nucleic acid linked to the mobile solid support,
the presence of the label indicating the polymerization of the test nucleic acid to the second nucleic acid and ligation of the labeled third nucleic acid to the second nucleic acid linked to the mobile solid support, and the identity of the test nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

20 25. A method of quantitating expression of a selected nucleic acid in a sample comprising

- a) contacting (i) message nucleic acid isolated from a selected source with (ii) a nucleic acid probe linked at its 5' end to a detectably tagged mobile solid support, wherein the nucleic acid probe comprises a region complementary to a section of the selected nucleic acid;
- 5 b) performing the detection method of any of claims 1, 13, 17, 19, 21, 22, 23, and 24; and
- c) quantitating the fluorescence linked to the mobile solid support, the quantity of fluorescence indicating the quantity of the selected nucleic acid in the sample.

26. The method of claim 25, wherein the message nucleic acid is mRNA, cRNA or

10 cDNA.

27. A method of detecting a result from an identification reaction to identify a selected nucleotide in a target nucleic acid comprising:

- a) contacting a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the second complementarity region is 5' of the first complementarity region and wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide, with a sample comprising the
- 15 target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a first hybridization product;

- b) performing a selected identification reaction with the first hybridization product to determine the identity of the selected nucleotide wherein a selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;
- 5 c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form a second hybridization product; and
- 10 d) detecting and/or identifying the label of the labeled detection product in the second hybridization product,
the presence and or identity of the label indicating the identity of the selected nucleotide in the target nucleic acid.

15 28. The method of claim 27, wherein the identification reaction is an oligonucleotide ligation reaction.

29. The method of claim 27, wherein the identification reaction comprises performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension; and wherein the detection comprises detecting the presence or absence 20 of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled nucleotide into the first hybridization product, and the identity of the incorporated labeled nucleotide

indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

30. The method of claim 27, wherein the target nucleic acid is an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a 5 cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.
31. The method of claim 27, wherein the mobile solid support is a bead.
32. The method of claim 31, wherein the bead is a polystyrene-divinylbenzene.
- 10 33. The method of claim 27, wherein the mobile solid support is detectably tagged with dye, a radiolabel, or a magnetic tag.
34. The method of claim 27, wherein the first nucleic acid is an amplification product.
35. The method of claim 27, wherein the first nucleic acid is a PCR product.
- 15 36. The method of claim 27, wherein the detecting is performed by passing the mobile solid support over a laser detection device capable of detecting/distinguishing the detectable tag.
37. The method of claim 27, wherein the detecting is performed by fixing the mobile solid support onto a two-dimensional surface and passing a laser detection device capable of detecting/distinguishing the detectable tag over the solid support.
- 20 38. A method of detecting a reaction product to identify a selected nucleotide in a target nucleic acid comprising:
 - a) contacting a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region comprises the oligonucleotide primer and the

second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and wherein the oligonucleotide primer comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide, with a sample comprising the target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a 5 first hybridization product;

10 b) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;

15 c) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form 20 an isolated second hybridization product; and

d) detecting the presence or absence of a label in the isolated second hybridization product, the presence of a label indicating the incorporation of the labeled nucleotide into the primer extension product, and the identity of the identified incorporated labeled nucleotide indicating the

identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

39. The method of claim 38, wherein the target nucleic acid is an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a 5 cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.
40. The method of claim 39, wherein the labeled chain -terminating nucleotide is a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a 10 dideoxynucleotide.
41. The method of claim 39, wherein the chain terminating nucleotide is a dideoxynucleotide and the primer extension is performed in the presence of one labeled, identified dideoxynucleotide and three different, non-labeled dideoxynucleotides.
42. The method of claim 39, wherein the chain terminating nucleotide is a 15 dideoxynucleotide, wherein the primer extension is performed in the presence of a first identified dideoxynucleotide labeled with a first detectable label, a second identified dideoxynucleotide labeled with a second detectable label, a third identified dideoxynucleotide labeled with a third detectable label and a fourth identified dideoxynucleotide labeled with a fourth detectable label, and wherein 20 detection of the presence of the first, the second, the third or the fourth detectable label in the second hybridization product indicates the identity of the nucleotide complementary to the selected nucleotide as the first, the second, the third or the fourth dideoxynucleotide, respectively.

43. The method of claim 39, wherein the mobile solid support is a bead.
44. The method of claim 43, wherein the bead is a polystyrene-divinylbenzene.
45. The method of claim 43, wherein the mobile solid support is detectably tagged with dye, a radiolabel, or a magnetic tag.
- 5 46. The method of claim 39, wherein the target nucleic acid is an amplification product.
47. The method of claim 39, wherein the target nucleic acid is a PCR product.
48. The method of claim 39, wherein the detecting is performed by passing the mobile solid support over a laser detection device capable of detecting/distinguishing the 10 detectable label.
49. The method of claim 39, wherein the detecting is performed by fixing the mobile solid support onto a two-dimensional surface and passing a laser detection device capable of detecting/distinguishing the detectable label over the solid support.
50. A method of detecting a result from an identification reaction to identify a 15 selected nucleotide in a target nucleic acid comprising:
 - a) hybridizing (i) a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide and the second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and (ii) a 20 fluorescently labeled reporter oligonucleotide comprising a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected nucleotide, to a sample comprising the target

nucleic acid, under hybridization conditions that allow specific hybridization between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide and that also allow specific hybridization between the reporter oligonucleotide and the section of the target nucleic acid complementary to the reporter oligonucleotide, to form a first hybridization product that defines a gap opposite the selected nucleotide;

5 b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation to form a labeled product;

10 c) dissociating the hybridized nucleic acids;

d) isolating the labeled product by contacting the labeled product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form a second hybridization product; and

15 e) detecting the presence or absence of the label in the second hybridization product,

20 the presence of the label indicating polymerization of the identified test nucleotide to the target oligonucleotide and ligation of the labeled reporter oligonucleotide to the polymerized target oligonucleotide, and the identity of the identified test nucleotide indicating the identity of the nucleotide

complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

51. The method of claim 50, wherein the target nucleic acid is an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.
52. The method of claim 50, wherein the reporter oligonucleotide is selected from the group consisting of the 1024 member set of all possible 5-mer oligonucleotides.
- 10 53. The method of claim 50, wherein the mobile solid support is a bead.
54. The method of claim 53, wherein the bead is a polystyrene-divinylbenzene.
55. The method of claim 50, wherein the mobile solid support is detectably tagged with dye, a radiolabel, or a magnetic tag.
- 15 56. The method of claim 50, wherein the target nucleic acid is an amplification product.
57. The method of claim 50, wherein the target nucleic acid is a PCR product.
58. The method of claim 50, wherein the detecting is performed by passing the mobile solid support over a laser detection device capable of detecting/distinguishing the detectable label.
- 20 59. The method of claim 50, wherein the detecting is performed by fixing the mobile solid support onto a two-dimensional surface and passing a laser detection device capable of detecting/distinguishing the detectable label over the solid support.
60. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- a) performing an amplification of the genomic DNA using a first nucleic acid primer comprising a region complementary to a section of one strand of the nucleic acid that is 5' of the selected nucleotide, and a second nucleic acid primer complementary to a section of the opposite strand of the nucleic acid downstream of the selected nucleotide, under conditions for specific amplification of the region of the selected nucleotide between the two primers, to form a PCR product;
- 5 b) contacting the PCR product with a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions to form a first hybridization product;
- 10 c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;
- 15 d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;
- 20 e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation

of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

5 f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

10 61. The method of claim 60, wherein the labeled chain -terminating nucleotide is a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

15 62. The method of claim 60, wherein the DNA has been sheared to reduce viscosity.

16 63. The method of claim 60, wherein the DNA has been treated with DNase to reduce viscosity.

20 64. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of the nucleic acid that is directly 5' of the selected nucleotide, and using T7 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the second strand complementary to the cRNA strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;

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b) contacting the amplification product with a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions to form a first hybridization product;

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c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;

d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;

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e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

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f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the

non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

65. The method of claim 64, wherein the labeled chain -terminating nucleotide is a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a 5 dideoxynucleotide.

66. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- a) contacting the genomic DNA with a target oligonucleotide comprising a first complementarity region and a second complementarity region, 10 wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions for forming a specific first hybridization product;
- b) performing a primer extension reaction with the specific first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- c) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a 20 mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;

d) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide
5 indicating the identity of the nucleotide complementary to the selected nucleotide; and

e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.
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67. The method of claim 66, wherein the labeled chain-terminating nucleotide is a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

68. A method of identifying a selected nucleotide in a target nucleic acid comprising

15 a) contacting the target nucleic acid with

i. a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of the first nucleic acid that is directly 5' of the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in an identified test nucleotide positioned to base-pair with the selected nucleotide, and
20 wherein the second complementarity region is 5' to the first complementarity region, and

ii. a fluorescently labeled reporter oligonucleotide, wherein the reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide,

5 under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to hybridize and the target nucleic acid and the reporter oligonucleotide to hybridize, thus forming a first hybridization product;

b) adding to the first hybridization product a ligase under ligation conditions;

c) isolating the first hybridization product by contacting the first

10 hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

15 d) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product, the presence of the label indicating the ligation of the labeled reporter oligonucleotide to the target oligonucleotide, and the identity of the test nucleotide in the target oligonucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying 20 the selected nucleotide.

69. The method of claim 68, wherein the first nucleic acid is genomic DNA treated to reduce viscosity, an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.

70. A method of identifying a selected nucleotide in a target nucleic acid comprising

- a) contacting the target nucleic acid with
 - i. a target oligonucleotide linked at its 3' end to a fluorescent label, wherein the target oligonucleotide comprises a first complementarity region that is complementary to a section of the target nucleic acid that is directly 3' of the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and wherein the target oligonucleotide has a second complementarity region 5' of the first complementarity region, and
 - ii. a reporter oligonucleotide fluorescently labeled at its 5' end, wherein the reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to hybridize and the target nucleic acid and the reporter oligonucleotide to hybridize, to form a first hybridization product;
- b) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and
- d) detecting the presence or absence of fluorescent energy transfer between the fluorescent label at the 3' end of the target oligonucleotide and the fluorescent label at the 5' end of the reporter oligonucleotide in the second hybridization product, the presence of fluorescent energy transfer indicating the hybridization of

the identified test nucleotide to the target nucleic acid, and the identity of the hybridized test nucleotide in the target oligonucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

5 71. A method of identifying a selected nucleotide in a target nucleic acid comprising

a) contacting the target nucleic acid with

- i. a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region is complementary to a section of the target nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
- ii. a reporter oligonucleotide fluorescently labeled, wherein the reporter oligonucleotide comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,

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under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to form a hybridization product and the target nucleic acid and the reporter oligonucleotide to form a hybridization product, wherein the target nucleic acid, target oligonucleotide and reporter oligonucleotide form a hybridization product

20 that defines a gap opposite the selected nucleotide;

b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation;

c) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile

solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

5 d) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product, the presence of the label indicating the polymerization of the test nucleic acid to the target oligonucleotide and ligation of the labeled reporter oligonucleotide to the target oligonucleotide linked to the mobile solid support, and the identity of the test

10 nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

72. A method of quantitating expression of a selected nucleic acid in a sample comprising

15 a) contacting (i) message nucleic acid isolated from a selected source with (ii) a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region comprises a region complementary to a section of the selected nucleic acid;

20 b) performing a selected identification reaction with the first hybridization product to determine the identity of the selected nucleotide wherein a selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;

25 c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support,

wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated hybridization product; and

- 5 d) quantitating the fluorescence in the isolated hybridization product, the quantity of fluorescence indicating the quantity of the selected nucleic acid in the sample.

73. The method of claim 72, wherein the message nucleic acid is mRNA, cRNA or cDNA.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/13928

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 19/34; C12Q 1/68
US CL : 435/91.2, 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.2, 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, CAPLUS, MEDLINE, LIFESCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,E	WO 99/31272 A1 (THE GENERAL HOSPITAL CORPORATION) 24 June 1999.	1-72
Y	US 5,679,524 A (NIKIFOROV et al.) 21 October 1997, col.5, lines 55-67, col.6 lines 1-65, col. 9 lines 39-67, col.10, lines 21-38, col.11 lines 6-9.	1-10, 13-35, 38-47, 49-57, 60-72
Y	US 4,988,617 A (LANDEGREN et al.) 29 January 1991, col. 2 lines 34-68, col.3 lines 1-53, col. 6 lines 38-53, col.8 lines 39-69, col. 11 lines 45-68.	1-10, 13-35, 38-47, 49-57, 60-72
A	US 5,610,287 A (NIKIFOROV et al.) 11 March 1997.	1-72
A,P	US 5,811,239 A (FRAYNE et al) 22 September 1998.	1-72

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

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01 SEPTEMBER 1999

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/13928

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,919,626 A (SHI et al.) 06 July 1999.	1-72
Y	SCHNEIDER-STOCK et al. Improved detection of p53 mutations in soft tissue tumors using new gel composition for automated nonradioactive analysis of single-strand conformation polymorphism. Electrophoresis. 1997, Vol.18, pages 2849-2851, see entire document.	11-12, 36-37, 48, 58-59,